SHORT COMMUNICATIONS

CHANGES IN THE FORMONONETIN CONTENT OF DETACHED MATURE LEAVES OF *TRIFOLIUM SUBTERRANEUM**

By R. C. Rossiter†

Anthocyanin formation is stimulated in the leaves of some plants when the leaves are detached and floated on water or on sucrose solutions and exposed to light (e.g. Eberhardt 1954; Creasy, Maxie, and Chichester 1965). The synthesis of other flavonoids, e.g. flavans, can be increased under these conditions (Creasy and Swain 1966).

We have observed that anthocyanin formation can be greatly stimulated by detaching fully expanded leaves of subterranean clover (*T. subterraneum*) and holding them for several days in diffuse daylight. We then wondered whether the oestrogenic isoflavones formononetin, genistein, and biochanin A, which occur in subterranean clover (Beck 1964), were also increased. If they were, the point would be of some interest, since *with entire plants* the net increase in the amounts of these three isoflavones after the leaves reach full expansion is negligible (Rossiter and Beck 1967).

Several simple experiments were done with detached leaves, mostly using the Northam A strain of *T. subterraneum*. Increases in biochanin A were usually small, while for genistein the increases were seldom more than 50% and usually much less than this. Results for these two compounds are not presented here, since treatment differences rarely reached statistical significance. However, for formononetin there were large relative increases, and these are reported below.

Materials and Methods

The clover plants were grown in shallow boxes in an open-sided glasshouse during the winter growing season (average day and night temperatures usually more than 15°C and 10°C, respectively). Mature leaves showing no visible senescence were harvested between 1100 and 1500 hr as required, mixed before sorting in a cold room, and weighed. Sufficient leaves were used for each sample to provide a total fresh weight of *c.* 500 mg. The leaves (laminae only) were floated on 30 ml of solution in covered Petri dishes (9 cm diameter). In all instances the dry matter content of the leaves was within the range 20–25%.

Unless specified otherwise, incubations were done in diffuse daylight near a laboratory window where the mean temperature was 21°C, and the range about ±2°C. Light intensity at the leaf surface seldom exceeded 350 f.c., as measured with an EEL light-meter. Some incubations were done in an artificially lit type LB cabinet (Morse and Evans 1962).

Formononetin was estimated by the thin-layer chromatographic method of Beck (1964) with modifications based on the microtechnique of Francis and Millington (1965).

In the first two experiments the treatments were unreplicated, but three replications were used thereafter. Isoflavone contents are expressed on the basis of original fresh weight, rather than per leaf, since leaf size varied between strains and between experiments.

* Manuscript received October 29, 1969.

† Division of Plant Industry, CSIRO, Western Australian Laboratories, Floreat Park, W.A. 6014.

Results and Discussion

Data for the first two experiments are summarized in Figure 1. Both for the Northam A strain and for Dwalganup—normally low and high in formononetin content respectively—the amounts of formononetin were increased when the leaves were floated on water, and more so on sucrose solutions. For Dwalganup the maximum increase was about threefold, and for Northam A, 30-fold. Sucrose at a concentration of 5% was less effective than at higher concentrations, and subsequent experiments were done with either 9 or 10% solutions. The results suggest that formononetin content was slightly higher on the twelfth than on the fourth day of incubation. There was no macroscopic evidence of microbial contamination, even on the twelfth day, but in subsequent experiments a 6- or 7-day period was used to reduce this risk.

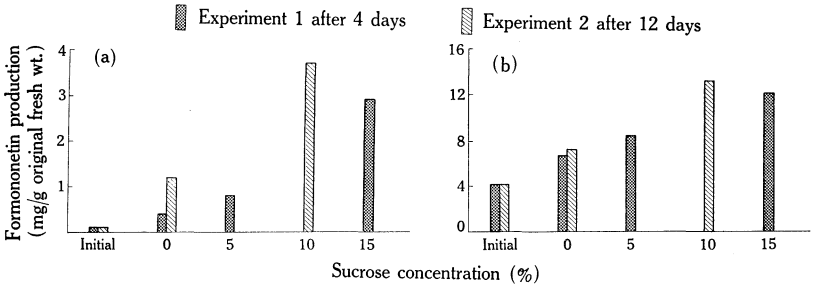

Fig. 1.—Increases in amounts of formononetin produced after excising leaves of Northam (*a*) and Dwalganup (*b*) strains and floating on water or sucrose solutions of various concentrations.

Three other strains of *T. subterraneum* were tested in another experiment over 7 days. The amounts of formononetin (mg/g original fresh weight) were as follows:

Strain	Initial	10% Sucrose
Daliak	0·4	1·7
Yarloop	6·5	13·1
Mt. Barker	0·14	5·8

The increases in formononetin content for the Northam A and Dwalganup strains of clover have been substantiated under controlled-environment conditions at higher light intensity, viz. 2000 f.c., and also at low light intensity (60–65 f.c.). Continuous light was used in the latter instance and there was little difference between fluorescent and incandescent light sources. Even in continuous darkness there was some stimulation of formononetin production—for example in leaves of the Daliak strain kept in continuous darkness at 21°C for 7 days, formononetin levels increased from an initial value of $0 \cdot 26 \pm 0 \cdot 01$ mg per gram original fresh weight to $0 \cdot 51 \pm 0 \cdot 02$ mg in the absence of sucrose and to $1 \cdot 3 \pm 0 \cdot 05$ mg in the presence of 10% sucrose. In diffuse daylight in the presence of 10% sucrose, levels of $3 \cdot 4 \pm 0 \cdot 24$ mg were attained.

Subsequent results have shown that DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] at 3×10^{-5}M prevented increases in formononetin, even in the presence of sucrose; that urea at 0·05M increased the level of formononetin in the presence of

sucrose; and that kinetin at $2{\cdot}5 \times 10^{-5}$M caused a slight fall in formononetin level, again in the presence of sucrose.

These experiments have shown that oestrogenic isoflavones, particularly formononetin, increase several fold when detached healthy mature leaves of *T. subterraneum* are floated on water or on sucrose solutions in the light; smaller increases were found in the dark. It is known that detached leaves of some plants, when floated on water in the light, exhibit increased activity of glucose-6-phosphate dehydrogenase (Farkas *et al.* 1964; Lewington, Talbot, and Simon 1967) and also of phenylalanine ammonia-lyase (Creasy 1968; Engelsma 1968; Zucker 1969). These two enzymes are associated with the synthesis of phenolic compounds. We now intend to examine the effect of detachment on phenylalanine ammonia-lyase in leaves of *T. subterraneum*.

Acknowledgments

I wish to thank Messrs. L. Klein and M. Jefferson for technical assistance with the experimental part of this investigation.

References

Beck, A. B. (1964).—*Aust. J. agric. Res.* **15**, 223–30.
Creasy, L. L. (1968).—*Phytochemistry* **7**, 1743–9.
Creasy, L. L., Maxie, E. C., and Chichester, C. O. (1965).—*Phytochemistry* **4**, 517–21.
Creasy, L. L., and Swain, T. (1966).—*Phytochemistry* **5**, 501–9.
Eberhardt, F. (1954).—*Planta* **43**, 253–87.
Engelsma, G. (1968).—*Planta* **82**, 355–68.
Farkas, G. L., Dezsi, L., Horvath, M., Kisban, K., and Udvardy, J. (1964).—*Phytopath. Z.* **49**, 343–54.
Francis, C. M., and Millington, A. J. (1965).—*Aust. J. agric. Res.* **16**, 557–64.
Lewington, R. J., Talbot, M., and Simon, E. W. (1967).—*J. exp. Bot.* **18**, 526–34.
Morse, R. N., and Evans, L. T. (1962).—*J. agric. Engng Res.* **7**, 128–40.
Rossiter, R. C., and Beck, A. B. (1967).—*Aust. J. agric. Res.* **18**, 561–73.
Zucker, M. (1969).—*Pl. Physiol., Lancaster* **44**, 912–22.